Cytokine production induced by non-encapsulated and encapsulated Porphyromonas gingivalis strains

Kunnen, A.; Dekker, D.C.; van Pampus, M.G.; Harmsen, H.J.; Aarnoudse, J.G.; Abbas, F.; Faas, M.M.

Published in:
Archives of Oral Biology

DOI:
10.1016/j.archoralbio.2012.07.013

Publication date:
2012

Available online at www.sciencedirect.com

SciVerse ScienceDirect

journal homepage: http://www.elsevier.com/locate/aob

Cytokine production induced by non-encapsulated and encapsulated *Porphyromonas gingivalis* strains

Alina Kunnen [a,e,*], *Daphne C. Dekker* [b], *Maria G. van Pampus* [c], *Hermie J.M. Harmsen* [d], *Jan G. Aarnoudse* [c], *Frank Abbas* [a], *Marijke M. Faas* [b]

[a] *Department of Periodontology, Center for Dentistry and Oral Hygiene, University Medical Center Groningen and University of Groningen, The Netherlands*

[b] *Division of Medical Biology, Department of Pathology and Medical Biology, University Medical Center Groningen and University of Groningen, The Netherlands*

[c] *Department of Obstetrics and Gynecology, University Medical Center Groningen and University of Groningen, The Netherlands*

[d] *Department of Medical Microbiology, University Medical Center Groningen and University of Groningen, The Netherlands*

[e] *Research and Innovation Group in Health Care and Nursing, Hanze University of Applied Sciences Groningen, The Netherlands*

ARTICLE INFO

Article history:
Accepted 27 July 2012

Keywords:
Periodontal disease
Monocytes
Cytokines
Lipopolysaccharides
Escherichia coli
P. gingivalis

ABSTRACT

Objective: Although the exact reason is not known, encapsulated gram-negative *Porphyromonas gingivalis* strains are more virulent than non-encapsulated strains. Since difference in virulence properties may be due to difference in cytokine production following recognition of the bacteria or their products by the host inflammatory cells, we compared cytokine production following stimulation with bacteria or lipopolysaccharides (LPS) of a non-encapsulated and an encapsulated *P. gingivalis* strain (K^- and K1).

Design: Tumour necrosis factor-alpha (TNF-α) production following stimulation of the cell-line Mono Mac 6 with bacteria or LPS of both *P. gingivalis* strains was determined using flow cytometry. Furthermore, we investigated the effects of the two *P. gingivalis* strains or their LPS on TNF-α and Interleukin (IL-1β, IL-6, IL-12 and IL-10) production in whole blood using Luminex. In both experiments, *Escherichia coli* bacteria and LPS were used as a reference.

Results: Both *P. gingivalis* strains induced lower cytokine production than *E. coli* with the exception of IL-6. *P. gingivalis* K1 bacteria elicited a higher overall cytokine production than *P. gingivalis* K^-. In contrast, *P. gingivalis* K1 LPS stimulation induced a lower cytokine production than *P. gingivalis* K^- LPS.

Conclusions: Our findings suggest that the encapsulated *P. gingivalis* K1 bacteria induce higher cytokine production than the non-encapsulated *P. gingivalis* K^-. This was not due to its LPS. The stronger induction of cytokines may contribute to the higher virulence of *P. gingivalis* K1.

1. Introduction

Periodontitis is a multifactorial, polymicrobial infection of the tissues surrounding the teeth, caused by a mixed microflora consisting of gram-negative and gram-positive micro-organisms. It is a chronic inflammatory disease involving complex interactions between the micro-organisms and immune response of the host and is characterised by collagen destruction and alveolar bone resorption.[1]

* *Corresponding author at*: University Medical Center Groningen, Center for Dentistry and Oral Hygiene, A. Deusinglaan 1, 9713 AV Groningen, The Netherlands. Tel.: +31 50 3633165; fax: +31 50 3632696.
E-mail address: a.kunnen@umcg.nl (A. Kunnen).

0003–9969/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.archoralbio.2012.07.013

An important gram-negative strict anaerobic micro-organism associated with periodontal breakdown and with the capacity to modulate inflammatory responses is *P. gingivalis*.[2,3] Several encapsulated and non-encapsulated serotypes of *P. gingivalis* have been described.[4–6] Encapsulated *P. gingivalis* serotypes are more virulent in experimental infections and cause a more invasive type of infection, whereas non-encapsulated *P. gingivalis* cause a more localised infection.[7] The exact reason for the higher virulence of encapsulated strains is not known, but may result from different cytokine production following recognition of the encapsulated or non-encapsulated bacteria by the host immune system.[8,9] *P. gingivalis* strains contain a variety of components on their cell surface, like lipopolysaccharides (LPS), lipoproteins and fimbriae, as well as capsular components, which may activate inflammatory cells, such as monocytes, and induce cytokine production.[2] Especially LPS is known as a potent stimulator of the host inflammatory response[10] and is thought to be a main virulence factor.[2] Therefore, we hypothesised that encapsulated and non-encapsulated *P. gingivalis* do induce different production of cytokines upon stimulation of inflammatory cells and that this may be due to their LPS.

Monocytes are the main regulators of the inflammatory response[11] by their ability to recognise bacteria and their products by pattern recognition receptors (PRRs), like Toll-like receptors (TLRs) and NOD-like receptors (NLRs).[12] Upon recognition of bacteria or their products by these PRRs, monocytes start producing cytokines.[13] In the present study, we investigated whether encapsulated and non-encapsulated *P. gingivalis* strains induced different cytokine production in monocytes. First we used a monocyte cell line, Mono Mac 6 (MM6), to study whether there are differences in cytokine responses of monocytes per se to the encapsulated and non-encapsulated *P. gingivalis* strains. We used TNF-α as a marker of cytokine production, since TNF-α is the most important regulatory pro-inflammatory cytokine, which is first produced by monocytes/macrophages upon bacterial or LPS stimulation.[14] Since this experiment showed different TNF-α responses to especially the LPS of the two *P. gingivalis* strains, in a second experiment, we incubated whole blood to simulate the natural environment,[15] with bacteria or LPS and measured production of the pro-inflammatory cytokines TNF-α, IL-1β, IL-6, IL-12 and the anti-inflammatory cytokine IL-10. In both experiments, we used *E. coli* bacteria or its LPS as a reference, since this species is known to be a strong inducer of pro-inflammatory cytokines.[16,17]

2. Materials and methods

2.1. Experimental design

Experiment 1: The first aim was to compare the pro-inflammatory properties of *P. gingivalis* K^-, *P. gingivalis* K1 and *E. coli* bacteria on monocytes per se. Therefore, we first evaluated the production of TNF-α after stimulation of MM6 cells[18] with the bacteria using flow cytometry. To study whether differences in virulence are the result of differences in pro-inflammatory potency of their LPS, we also determined TNF-α production by MM6 cells after stimulation with LPS of *P. gingivalis* (K^- and K1) as well as with *E. coli* LPS.

Experiment 2: Since in experiment 1 we found differences in TNF-α production by MM6 cells after stimulation with the LPS of the two *P. gingivalis* strains, we continued to study the production of other pro- and anti-inflammatory cytokines, now using whole blood, which simulates the in vivo situation better. For this experiment, whole blood of 15 healthy individuals was stimulated with bacteria as well as with LPS of *P. gingivalis* K^-, *P. gingivalis* K1 and *E. coli*. The production of TNF-α, IL-1β, IL-6, IL-12 and IL-10 was measured using the Luminex system.

2.2. Bacterial strains and growth conditions

E. coli ATCC 25922 was grown on 5% sheep blood agar plates (Mediaproducts, Groningen, The Netherlands) in air with 5% carbon dioxide (CO_2) at 37 °C for 1 day. Bacterial strains of *P. gingivalis* ATCC 33277 (K^-), which has been shown to be non-encapsulated[19] and *P. gingivalis* W50 (K1), which has been shown to have a capsule,[6,20] were generously provided by A.J. van Winkelhoff (Department of Oral Microbiology, Academic Centre for Dentistry Amsterdam, The Netherlands). *P. gingivalis* K^- and K1 were grown on Brucella blood agar (Mediaproducts, Groningen, The Netherlands), supplemented with 5% sheep blood, 5 mg/l hemin and 1 mg/l menadione in an anaerobic chamber with an atmosphere of 5% CO_2, 10% H_2 and 85% N_2 at 37 °C. After 4–7 days, one bacterial colony per strain was incubated in Todd–Hewitt broth (BBL Microbiology Systems), supplemented with hemin (5 mg/l), menadione (5 mg/l) and glucose (2 g/l) for one week. The bacterial cultures were harvested by centrifugation at 2773 × *g* for 10 min at 4 °C. The supernatant was decanted and the bacterial pellet was washed twice in phosphate-buffered saline (PBS). The number of bacteria was evaluated by means of a microscope after gram-staining and resuspended in PBS at a number corresponding to approximately 1×10^8 bacteria/ml. All cultures were stored at −20 °C until used.

Lipopolysaccharides derived from *E. coli* 055:B5 (BioWhittaker, Walkersville, MD, USA); *P. gingivalis* ATCC 33277 (Ultra-Pure, Cat. #: tlrl-pglps, Lot. #: 28-06-PGLPS, InvivoGen, San Diego, USA) and *P. gingivalis* W50, a generous gift from M.A. Curtis (The Institute of Cell and Molecular Science, Barts and The London School of Medicine and Dentistry, UK) were used.

2.3. Cell line and culture

The MM6 cell line was cultured in RPMI (Complete Roswell Park Memorial Institute) 1640 medium (Invitrogen, California, USA) with 10% foetal calf serum (FCS), supplemented with 1% L-glutamine, 1% Na-pyruvate, 0.1% BME (2β-mercaptoethanol), 0.6% gentamycin sulfate and 0.05% fungizone (amfotericine B). Cell-suspensions were cultured at 37 °C with a 5% CO_2 humidified atmosphere and diluted 1:5 in the culture medium for every 3–4 days.

2.4. Experiment 1: TNF-α production after stimulation of MM6 cells

Before stimulation, MM6 cells were counted and diluted in RPMI to a cell concentration of 4×10^9 cells/l. 2×10^8 MM6 cells/l were

supplemented with 9% FCS and 2 ng/ml monensin (Sigma Aldrich, St. Louis, MO, USA) to inhibit intracellular transport of TNF-α through the ER–Golgi complex.[21] Immediately prior to use, the stocks of 1×10^8 bacteria/ml of *E. coli*, *P. gingivalis* K$^-$ and *P. gingivalis* K1 were thawed and used undiluted or diluted in PBS (10, 100 and 1000 times). 250 μl of bacterial suspensions was added to 50 μl of the MM6 suspension to reach a final bacterial concentration in the tubes of 8.3×10^7, 8.3×10^6, 8.3×10^5 and 8.3×10^4 bacteria/ml respectively. All cultures were incubated for 4 h at 37 °C in a 5% CO_2 humidified atmosphere. Similarly, MM6 cells were incubated with increasing concentrations (0.005, 0.05, 0.25, 0.5, 1.0, 1.5 and 2.5 μg/ml) of *P. gingivalis* K$^-$ LPS, *P. gingivalis* K1 LPS or with *E. coli* LPS. For negative control (unstimulated MM6 cells), MM6 cells were incubated in the absence of bacterial or LPS challenge.

2.4.1. *Sample labelling*

After incubation, 200 μl of both stimulated and unstimulated MM6 cells were fixed in 1 ml 2% paraformaldehyde (PFA) in PBS for 5 min. Then, after centrifugation at 467 × *g* for 5 min, the pellet was resuspended in 1 ml 0.1% saponin solution (Sigma Aldrich, St. Louis, MO, USA) in washing buffer [PBS with 0.5% bovine serum albumin (BSA) and 0.1% sodium azide] for 5 min in order to permeabilise the MM6 cells. After centrifugation (5 min at 467 × *g*) and aspiration, the cells were incubated with PE-labelled mouse-anti-human TNF-α antibody (BD Pharmingen, San Diego, USA), 1:10 diluted with 0.1% saponin solution in washing buffer for 30 min at room temperature in the dark. The MM6 cells were then washed again with 1 ml 0.1% saponin solution in washing buffer, and after centrifugation and aspiration, the cells were fixed with 200 μl 0.5% PFA in PBS and were kept in the dark at 4 °C until measured by flow cytometry, within 24 h after labelling.

2.4.2. *Flow cytometry*

MM6 cells were analysed using the FACS Calibur flow cytometer (Becton Dickinson, NJ, USA). Flow cytometry results were analysed using Winlist 6.0 software (Verity Software House, Topsham, ME, USA).

2.4.3. *Intracellular cytokines*

Five thousand MM6 cells were acquired by live gating on the total MM6 population using forward- and sidescatter characteristics. This gate was copied to a single parameter histogram, to determine intracellular TNF-α production of the MM6 cells. Using the unstimulated control sample, a linear gate was set in the histogram so that at least 99% of the unstimulated MM6 cells were negative for TNF-α production. This gate was then copied to the histogram for stimulated MM6 cells. The percentage of positive cells was evaluated from the histogram of the stimulated cells. Results are expressed as percentage of TNF-α positive cells.

2.5. *Experiment 2: cytokine production after stimulation of whole blood*

Whole blood samples were obtained by venous puncture from 15 healthy females. Protocols for this study were approved by the local ethics committee and a written informed consent was obtained from each subject before participation. Since males and females do respond differently to endotoxin,[22] in order to obtain a homogenous study-population, only Caucasian women between 20 and 40 years of age, with no known systemic diseases, were included in this study. Moreover, female hormonal fluctuations during the different phases of the ovarian cycle influence the sensitivity of monocytes to endotoxin,[23] therefore, for all individuals, blood was drawn in the follicular phase of the menstrual cycle. Immediately after blood sampling, all participants were submitted to a periodontal examination by a certified dental hygienist (A.K.) using the Dutch Periodontal Screening Index (DPSI) to establish the periodontal condition.[24] To avoid the risk of including participants with a generalised inflammatory response due to periodontitis, we excluded subjects with a DPSI score of 3+ or 4, which is indicative for destructive periodontal disease.[24] Blood samples (10 ml) were collected in vacutainer tubes containing lithium heparin (Becton Dickinson, Rutherford, NJ, USA). The stock bacterial cultures were thawed. Immediately after sampling, 250 μl of undiluted stock bacterial cultures of *E. coli*, *P. gingivalis* K$^-$ or *P. gingivalis* K1 were mixed with 250 μl of heparinised blood (final numbers: 5×10^7 bacteria/ml). Furthermore, 250 μl of heparinised whole blood was mixed with 250 μl RPMI and LPS of *E. coli*, *P. gingivalis* K$^-$ or *P. gingivalis* K1 was added (2 μg/ml). Negative controls were incubated in the absence of bacterial or LPS challenge under similar circumstances. All samples were incubated for 24 h at 37 °C in a 5% CO_2 humidified atmosphere. After stimulation, all samples were pipetted into 1.5 ml Eppendorf tubes and centrifuged for 10 min at 316 × *g* (4 °C). The plasma was then pipetted into new 1.5 ml Eppendorf tubes and centrifuged again for 5 min at 1972 × *g* (4 °C). Supernatants were frozen at −80 °C until cytokine concentrations were measured using Luminex.

2.5.1. *Determination of extracellular cytokine production in whole blood*

Cytokine levels in whole blood were measured using a Bio-Plex™ premixed cytokine assay, human 5-plex group I; Cat. #: M50019PLCW, control 5016683 (Bio-Rad Laboratories, Hercules, USA). This customised kit simultaneously measured human TNF-α, IL-1β, IL-6, IL-10 and IL-12 (p70). Standard curves for each cytokine were generated using the reference cytokine concentrations supplied in this kit. Assay buffer, plasma and duplex standards were pipetted into the wells according to the manufacturers' instruction manual. In brief, after prewetting the wells of a 96-well filter plate with assay buffer, 50 μl of coupled beads were added to the wells and washed twice with assay buffer using a vacuum manifold (Millipore, MA, USA). 50 μl of 1:3 diluted plasma (sample diluent supplied in the kit) and standards were pipetted into the wells and incubated for 30 min with the coupled beads. The wells were then washed three times and 25 μl of detection antibody was subsequently added. After 30 min incubation, the wells were again washed three times and incubated for 10 min with 50 μl streptavidin-PE. After 10 min of incubation, the wells were washed three times in order to remove the unbound streptavidin-PE. Finally, 125 μl of assay buffer was added to each well after which the beads were analysed using the Luminex LX100™ multiplex assay detection system. Raw data (mean fluorescence intensity, MFI) were analysed using STarStation V2.3.

2.6. Data analysis

Statistical analysis was performed using GraphPad Prism 5 for Windows (Graphpad Software, San Diego, CA, USA). Normality tests were performed using the Shapiro–Wilk test. Values were given as mean ± standard error of the mean (SEM) for normally distributed variables. Not normally distributed data were presented as box and whisker plots showing the median values, interquartile and full ranges of value. Outliers were defined as data points greater than 1.5 the interquartile range from the median value. To evaluate differences between the dose response curves of the various bacterial and LPS stimulations, we calculated the area under the curve (AUC). Statistical comparisons between the AUCs after the various bacterial and LPS stimulations were performed using the unpaired Student's *t*-test with Bonferroni's correction. Differences in cytokine concentrations in plasma after stimulation with the different bacterial species as well as after the different LPS stimulations were evaluated by using Wilcoxon's Signed Rank test with Bonferroni's correction. Pro- or anti-inflammatory ratios were calculated by dividing the pro-inflammatory cytokine production (TNF-α and IL-12) by the anti-inflammatory cytokine production (IL-10). The ratios between the various bacterial and LPS stimulations were evaluated by using Wilcoxon's Signed Rank test with Bonferroni's correction. For all experiments, a *p*-value of <0.05 was accepted as statistically significant.

3. Results

3.1. Experiment 1: TNF-α production after stimulation of MM6 cells

MM6 cells exposed to only medium showed no TNF-α producing MM6 cells. Stimulation with increasing numbers of bacteria of *P. gingivalis* K^- and *P. gingivalis* K1 showed a dose-dependent increase in the percentage of TNF-α positive monocytes (Fig. 1(a)). Differences in dose response curves after the various bacterial stimulations were calculated using the AUC (Fig. 1(a), inset). A significantly higher AUC was observed after stimulation with bacteria of *E. coli* as compared with *P. gingivalis* K^- and *P. gingivalis* K1 ($p < 0.05$). No differences in the AUC between *P. gingivalis* K^- and *P. gingivalis* K1 stimulation were observed.

The percentages of TNF-α positive MM6 cells increased after stimulation with 0.005 μg/ml *E. coli* LPS, and then remained constant following stimulation with higher concentrations (Fig. 1(b)). Stimulation with increasing concentrations of *P. gingivalis* K^- LPS and K1 LPS induced a dose-dependent increase of TNF-α positive MM6 cells, starting at concentrations higher than 0.05 μg/ml LPS. As can be depicted from Fig. 1(b) (inset), a significantly higher AUC was observed after *E. coli* LPS stimulation as compared with both *P. gingivalis* strains ($p < 0.05$). Also, a significantly higher AUC after stimulation with LPS of *P. gingivalis* K^- as compared with *P. gingivalis* K1 was observed ($p < 0.05$).

3.2. Experiment 2: cytokine production after stimulation of whole blood

No cytokines were detected in plasma from unstimulated blood samples (data not shown). Apart from IL-6 production after *P. gingivalis* K1 stimulation, stimulation with *E. coli* induced an overall higher cytokine production as compared with *P. gingivalis* K^- and *P. gingivalis* K1 stimulation ($p < 0.05$) (Fig. 2). Furthermore, stimulation with *P. gingivalis* K^- resulted in a significantly lower overall cytokine production than *P. gingivalis* K1 ($p < 0.05$, for all cytokines tested).

Cytokine production was higher after whole blood stimulation with *E. coli* LPS, as compared with the LPS of *P. gingivalis* K^- and *P. gingivalis* K1 ($p < 0.05$, for all cytokines tested) (Fig. 3). Stimulation with *P. gingivalis* K^- LPS induced a significant

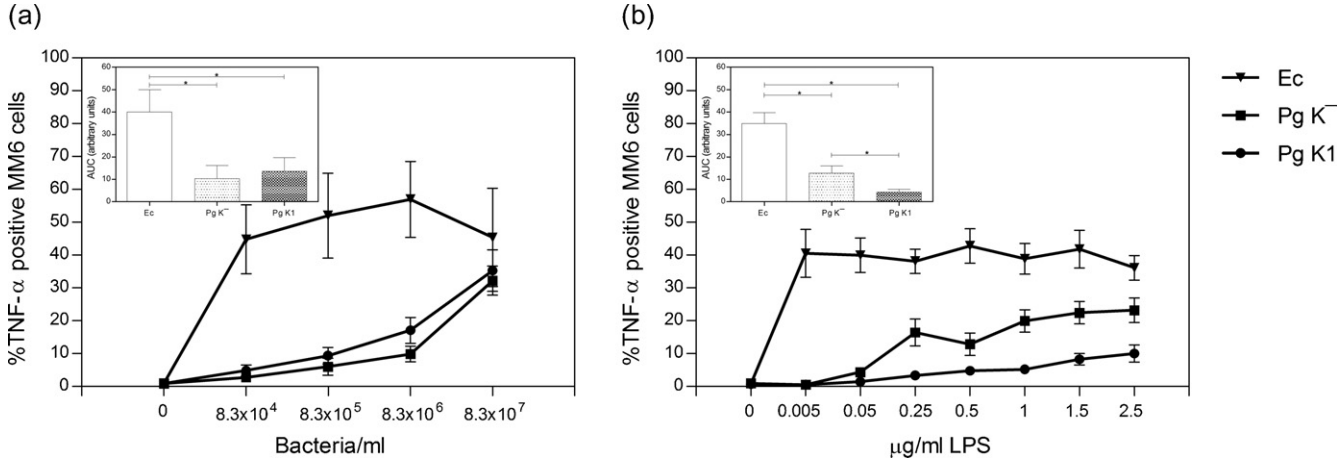

Fig. 1 – (a) Mean ± SEM percentages of TNF-α positive MM6 cells after stimulation with increasing numbers of bacteria from *P. gingivalis* K^- (Pg K^-) or *P. gingivalis* K1 (Pg K1), with *E. coli* (Ec) as reference strain and (b) mean ± SEM percentages of TNF-α positive MM6 cells after stimulation with increasing concentrations of LPS from Pg K^-, Pg K1 and from Ec as reference strain. Insets: mean area under the curve (AUC) ± SEM of TNF-α positive MM6 cells after stimulation with increasing numbers of bacteria from Pg K^-, Pg K1 and Ec, or with increasing concentrations of LPS from Pg K^-, Pg K1 and Ec. (*) Significantly different (unpaired Student's *t*-test with Bonferroni's correction; $p < 0.05$).

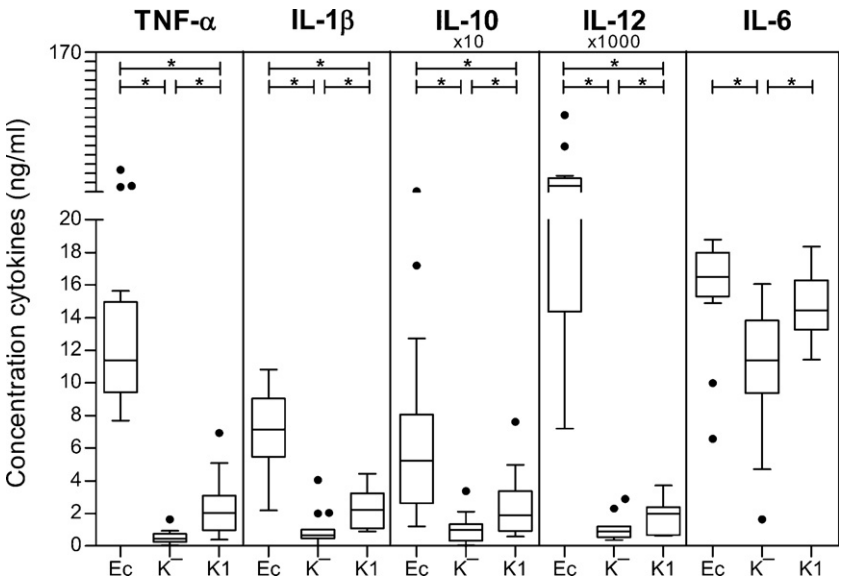

Fig. 2 – Median, 25th and 75th percentiles and 1.5 interquartile range (error bar) of secretion of TNF-α, IL-1β, IL-10, IL-12 and IL-6 in human whole blood following stimulation with 5×10^7 bacteria/ml of *E. coli* (Ec), *P. gingivalis* K⁻ (Pg K⁻) or *P. gingivalis* K1 (Pg K1). Outlier values are represented as individual points: ●. (*) Significantly different (Wilcoxon's Signed Rank test with Bonferroni's correction; $p < 0.05$).

higher overall cytokine production than *P. gingivalis* K1 LPS ($p < 0.05$, for all cytokines tested).

3.2.1. *Pro-/anti-inflammatory ratios*

We observed that cytokine production was induced differently following exposure to the various bacteria or LPS. Therefore, we determined the ratio between pro-inflammatory and anti-inflammatory cytokines after the various stimulations.

The TNF-α/IL-10 ratios after both *P. gingivalis* bacterial stimulations were lower than after *E. coli* stimulation ($p < 0.05$) (Fig. 4(a)). There were no differences in the TNF-α/IL-10 ratios between the two *P. gingivalis* bacterial strains. Fig. 4(b) shows the TNF-α/IL-10 ratios after the various LPS stimulations. No differences in the TNF-α/IL-10 ratios were observed between stimulation with LPS of *E. coli* as compared with both *P. gingivalis* strains. However, a higher TNF-α/IL-10 ratio was

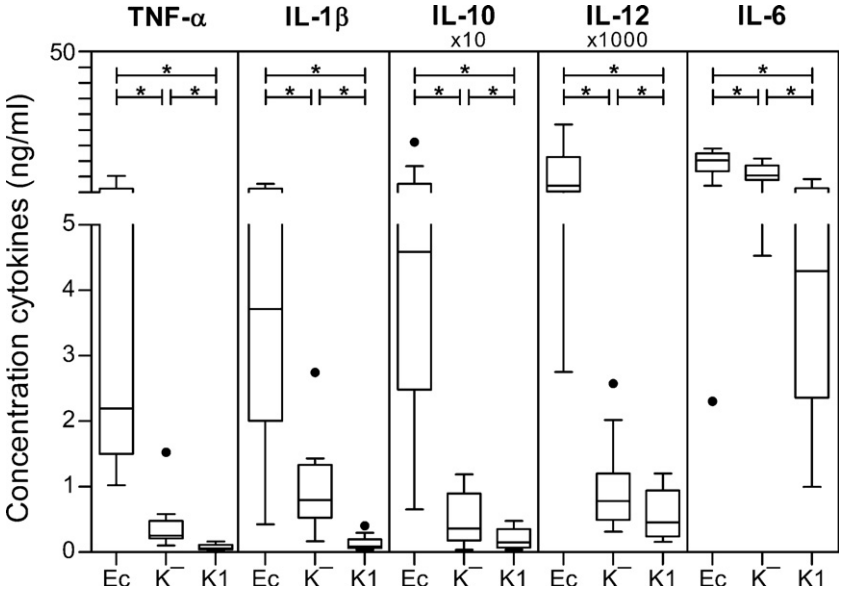

Fig. 3 – Median, 25th and 75th percentiles and 1.5 interquartile range (error bar) of secretion of TNF-α, IL-1β, IL-10, IL-12 and IL-6 in human whole blood following stimulation with 2 μg/ml LPS of *E. coli* (Ec), *P. gingivalis* K⁻ (Pg K⁻) or *P. gingivalis* K1 (Pg K1). Outlier values are represented as individual points: ●. (*) Significantly different (Wilcoxon's Signed Rank test with Bonferroni's correction; $p < 0.05$).

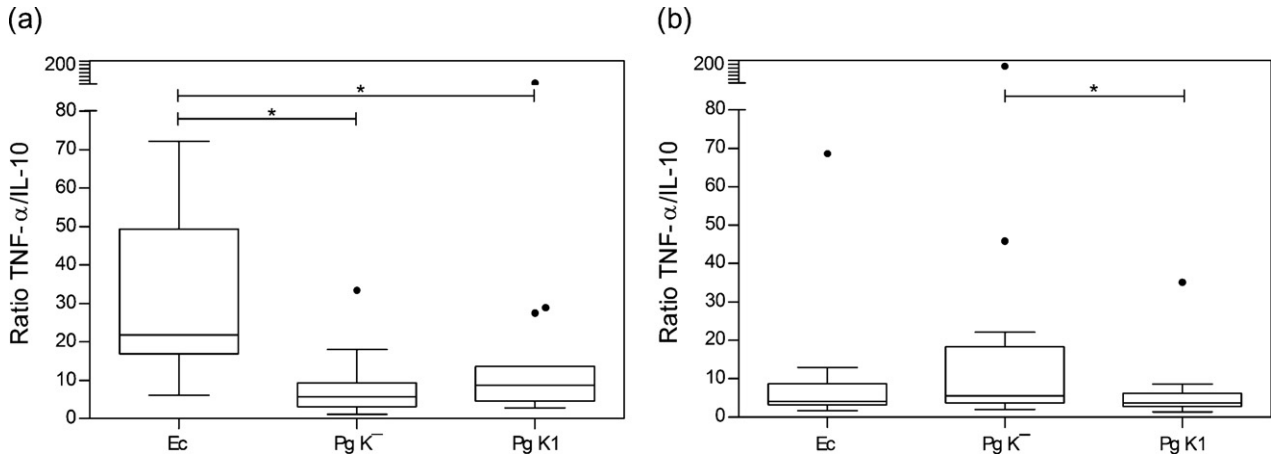

Fig. 4 – Median, 25th and 75th percentiles and 1.5 interquartile range (error bar) of TNF-α/IL-10 ratios: (a) following stimulation with 5×10^7 bacteria/ml of *E. coli* (Ec), *P. gingivalis* K⁻ (Pg K⁻) or *P. gingivalis* K1 (Pg K1) and (b) after stimulation with 2 μg/ml LPS of Ec, Pg K⁻ or Pg K1. Outlier values are represented as individual points: ●. (*) Significantly different (Wilcoxon's Signed Rank test with Bonferroni's correction; $p < 0.05$).

observed after *P. gingivalis* K⁻ LPS stimulation as compared with *P. gingivalis* K1 ($p < 0.05$).

IL-12 is an important pro-inflammatory cytokine associated with aggressive periodontal disease,[25] forming a link between innate and adaptive immunity.[26] Therefore, we calculated the ratios of IL-12/IL-10 after the various stimulations. A significantly higher IL-12/IL-10 ratio was seen after stimulation with *E. coli* bacterial stimulation as compared with both *P. gingivalis* strains ($p < 0.05$) (Fig. 5(a)). No significant differences in the IL-12/IL-10 ratios between the two *P. gingivalis* strains were observed after bacterial stimulation. Fig. 5(b) shows the IL-12/IL-10 ratios after the various LPS stimulations. There were no differences in the IL-12/IL-10 ratios between the various stimulations.

4. Discussion

This study was undertaken to investigate whether differences in virulence properties between *P. gingivalis* K⁻ and *P. gingivalis* K1 may be due to differences in their capacities to induce cytokine production by monocytes. As expected, we showed that bacteria and LPS of both *P. gingivalis* strains were less potent inducers of cytokines as compared with our reference strain *E. coli*. In line with our hypothesis, we found prominent differences in cytokine production following incubation with *P. gingivalis* K⁻ vs. *P. gingivalis* K1 bacteria or LPS. *P. gingivalis* K⁻ bacteria in general induced a lower cytokine production as compared with *P. gingivalis* K1. This difference cannot be due

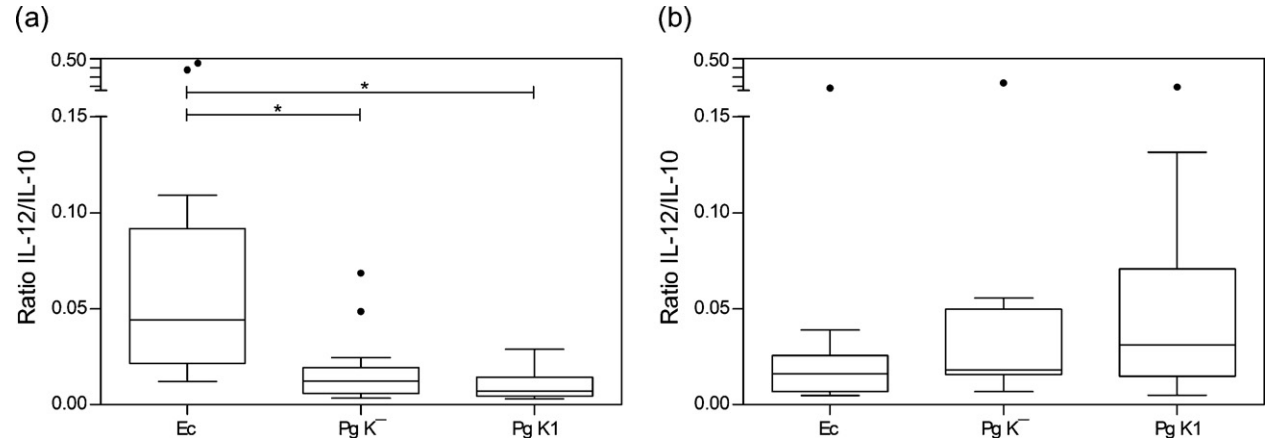

Fig. 5 – Median, 25th and 75th percentiles and 1.5 interquartile range (error bar) of IL-12/IL-10 ratios: (a) following stimulation with 5×10^7 bacteria/ml of *E. coli* (Ec), *P. gingivalis* K⁻ (Pg K⁻) or *P. gingivalis* K1 (Pg K1) and (b) after stimulation with 2 μg/ml LPS of Ec, Pg K⁻ or Pg K1. Outlier values are represented as individual points: ●. (*) Significantly different (Wilcoxon's Signed Rank test with Bonferroni's correction; $p < 0.05$).

to differences in cytokine stimulation by their LPS, since *P. gingivalis* K$^-$ LPS induced a higher cytokine production as compared with LPS of *P. gingivalis* K1.

The minor differences in results between experiments 1 and 2 can be explained by the use of different experimental protocols, i.e. use of MM6 vs. whole blood, use of intracellular cytokine production vs. extracellular production, 4 h stimulation vs. 24 h. Despite these minor differences, in both experiments, we found a higher overall cytokine production and higher TNF-α/IL-10 and IL-12/IL-10 ratio after *E. coli* bacterial stimulation as compared with *P. gingivalis* bacterial stimulation. This higher pro-inflammatory cytokine production following *E. coli* stimulation was expected, since it is a well-established fact that *E. coli* is a more potent inducer of pro-inflammatory cytokines than *P. gingivalis* both in vivo[17] and in vitro.[16] The production of IL-6, however, appeared to be much more similar between *E. coli* bacteria and the *P. gingivalis* species. This may have an in vivo relevance, since IL-6 plays an important role in regulating the immune response and leucocyte recruitment.[27] It also stimulates bone resorption by stimulating the formation and activation of osteoclasts.[28] IL-6 may therefore play an important role in the pathogenesis of periodontal diseases.

In addition, the overall decreased production of all cytokines by inflammatory cells after bacterial stimulation with *P. gingivalis* K$^-$ as compared with *P. gingivalis* K1 is in line with previous findings.[8,9] The higher total cytokine production may play a role in the more invasive character of the infection following *P. gingivalis* K1 infection in vivo. Increased cytokine production may enhance the spreading of the infection by activating other leucocytes in the environment and by attacking these cells to the site of infection.[28] Various bacterial substances may be responsible for the differences in cytokine induction after *P. gingivalis* K$^-$ compared with *P. gingivalis* K1 stimulation. The type of fimbriae of *P. gingivalis* K1 (type IV *fimA*) has been reported to induce a much stronger systemic inflammation in a mouse model as compared with the type of fimbriae of *P. gingivalis* K$^-$ (type 1 *fimA*).[29] Differences in cysteine proteinase structure may also have accounted for the observed variety in cytokine production between the two *P. gingivalis* strains.[30] Furthermore, the presence of capsular polysaccharides in the bacterial suspension of *P. gingivalis* K1 could have contributed to the observed differences between the bacterial strains. The capsule of *P. gingivalis* K1 has been shown to reduce phagocytosis in vivo, providing the bacterium with a mechanism to evade internalisation and clearance by host inflammatory cells.[31] A decreased phagocytosis of these bacteria may result in increased numbers of bacteria which can be recognised by monocytes, leading to increased cytokine production. However, recent studies looking at cytokine production by inflammatory cells after stimulation with encapsulated *P. gingivalis* and non-encapsulated mutants have shown higher cytokine production in the non-encapsulated mutant.[31,32] Additional in vivo studies are thus needed to elucidate the role of the capsule of *P. gingivalis* in host recognition and subsequent inflammatory responses.

Differences in chemical characterisation and biologic properties of the LPS[33] may also have played a role, since the LPS is considered as one of the most important pro-inflammatory molecules of gram-negative bacteria[2]. Although *E. coli* LPS and *P. gingivalis* LPS differ in chemical structure of the lipid A species,[34] and signal through different Toll-like receptors (TLR2 for LPS of *P. gingivalis*[35] and TLR4 for LPS of *E. coli*[36,37]), we found no differences in the IL-12/IL-10 and TNF-α/IL-10 ratio between *P. gingivalis* LPS vs. *E. coli* LPS. Our results are thus not in line with the suggestion that TLR2 is a weak inducer of pro-inflammatory cytokines.[38] It, however, corroborates the suggestion that TLR2 activation may also induce strong type 1 helper T cell (T_H1) responses[39] and indicates that TLR2 activation may induce pro-inflammatory or immunomodulatory signalling.[38,40]

From our results, it is unlikely that LPS of the *P. gingivalis* strains is responsible for the observed differences in cytokine production following stimulation with *P. gingivalis* K$^-$ and K1 bacteria. In contrast to *P. gingivalis* bacterial stimulation, we observed a higher overall cytokine production after incubation with *P. gingivalis* K$^-$ LPS versus incubation with *P. gingivalis* K1 LPS and a higher TNF-α/IL-10 ratio. Our results are in agreement with a study of Bramanti et al.[33] who also showed higher TNF-α and IL-1β production after stimulation of inflammatory cells with LPS of *P. gingivalis* K$^-$ as compared with LPS of *P. gingivalis* K1. This may be due to differences in the chemical properties of the LPS between these two *P. gingivalis* strains, since variations in carbohydrate and galactosamine composition and lipid A proportion between the strains have been observed.[33] Therefore, our findings do not support the idea that the LPS of *P. gingivalis* plays an important role in the observed variations in virulence properties between the species.[2] Other bacterial products than LPS seem to be responsible for the higher cytokine production following stimulation with *P. gingivalis* K1 bacteria compared with *P. gingivalis* K$^-$ bacteria.

In summary, although *E. coli* bacteria were more potent inducers of cytokine production in whole blood, we also observed differences in cytokine production after stimulation with *P. gingivalis* K$^-$ vs. *P. gingivalis* K1: stimulation of whole blood with the encapsulated *P. gingivalis* K1 bacteria resulted in an overall higher cytokine production. This may be related to the increased virulence of the encapsulated *P. gingivalis* strain, since a higher cytokine production may result in the attraction of more and other leucocytes and increase the spreading of the inflammatory response. The reason why *P. gingivalis* K1 induced a higher cytokine production remains elusive from the present study, since the most obvious cytokine inducing substance from gram-negative bacteria and thus *P. gingivalis*, its LPS, did not induce a higher cytokine production as compared with LPS from *P. gingivalis* K$^-$. Therefore, future studies need to be directed towards identifying this mechanism as well as towards revealing a causal relationship between the higher cytokine production of *P. gingivalis* K1 and its higher virulence properties.

Acknowledgements

Funding: This study was supported by a grant from the NVvP (Dutch Society of Periodontology) which was awarded to Prof. F. Abbas.

Competing interest: None declared.

Ethical approval: The protocol for the whole blood experiments was approved by the local ethics committee (METc UMCG, approval no. 2008/168).

REFERENCES

1. Haffajee AD, Socransky SS. Microbial etiological agents of destructive periodontal diseases. *Periodontology 2000* 1994;**5**:78–111.
2. Holt SC, Kesavalu L, Walker S, Genco CA. Virulence factors of *Porphyromonas gingivalis*. *Periodontology 2000* 1999;**20**:168–238.
3. Lamont RJ, Jenkinson HF. Life below the gum line: pathogenic mechanisms of *Porphyromonas gingivalis*. *Microbiology and Molecular Biology Reviews* 1998;**62**(4):1244–63.
4. Grenier D, Mayrand D. Selected characteristics of pathogenic and nonpathogenic strains of *Bacteroides gingivalis*. *Journal of Clinical Microbiology* 1987;**25**(4):738–40.
5. Laine ML, Appelmelk BJ, van Winkelhoff AJ. Prevalence and distribution of six capsular serotypes of *Porphyromonas gingivalis* in periodontitis patients. *Journal of Dental Research* 1997;**76**(12):1840–4.
6. van Winkelhoff AJ, Appelmelk BJ, Kippuw N, de Graaff J. K-antigens in *Porphyromonas gingivalis* are associated with virulence. *Oral Microbiology and Immunology* 1993;**8**(5):259–65.
7. Laine ML, van Winkelhoff AJ. Virulence of six capsular serotypes of *Porphyromonas gingivalis* in a mouse model. *Oral Microbiology and Immunology* 1998;**13**(5):322–5.
8. Bodet C, Chandad F, Grenier D. *Porphyromonas gingivalis*-induced inflammatory mediator profile in an ex vivo human whole blood model. *Clinical and Experimental Immunology* 2006;**143**(1):50–7.
9. Vernal R, Leon R, Silva A, van Winkelhoff AJ, Garcia-Sanz JA, Sanz M. Differential cytokine expression by human dendritic cells in response to different *Porphyromonas gingivalis* capsular serotypes. *Journal of Clinical Periodontology* 2009;**36**(10):823–9.
10. Holt SC, Ebersole JL. *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*: the ''red complex'', a prototype polybacterial pathogenic consortium in periodontitis. *Periodontology 2000* 2005;**38**:72–122.
11. Agarwal S, Piesco NP, Johns LP, Riccelli AE. Differential expression of IL-1 beta, TNF-alpha, IL-6, and IL-8 in human monocytes in response to lipopolysaccharides from different microbes. *Journal of Dental Research* 1995;**74**(4): 1057–65.
12. Kumar H, Kawai T, Akira S. Pathogen recognition by the innate immune system. *International Reviews of Immunology* 2011;**30**(1):16–34.
13. Kumar H, Kawai T, Akira S. Pathogen recognition in the innate immune response. *Biochemical Journal* 2009;**420**(1): 1–16.
14. Abbas AK, Lichtman AH. *Basic immunology*. 2nd ed. 2006. p. 21–39.
15. De Groote D, Zangerle PF, Gevaert Y, Fassotte MF, Beguin Y, Noizat-Pirenne F, et al. Direct stimulation of cytokines (IL-1 beta, TNF-alpha, IL-6, IL-2, IFN-gamma and GM-CSF) in whole blood. I. Comparison with isolated PBMC stimulation. *Cytokine* 1992;**4**(3):239–48.
16. Darveau RP, Cunningham MD, Bailey T, Seachord C, Ratcliffe K, Bainbridge B, et al. Ability of bacteria associated with chronic inflammatory disease to stimulate E-selectin expression and promote neutrophil adhesion. *Infection and Immunity* 1995;**63**(4):1311–7.
17. Pulendran B, Kumar P, Cutler CW, Mohamadzadeh M, Van Dyke T, Banchereau J. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. *Journal of Immunology* 2001;**167**(9):5067–76.
18. Ziegler-Heitbrock HW, Thiel E, Fütterer A, Herzog V, Wirtz A, Riethmüller G. Establishment of a human cell line (Mono Mac 6) with characteristics of mature monocytes. *International Journal of Cancer* 1988;**41**(3):456–61.
19. Davey ME, Duncan MJ. Enhanced biofilm formation and loss of capsule synthesis: deletion of a putative glycosyltransferase in *Porphyromonas gingivalis*. *Journal of Bacteriology* 2006;**188**(15):5510–23.
20. Aduse-Opoku J, Slaney JM, Hashim A, Gallagher A, Gallagher RP, Rangarajan M, et al. Identification and characterization of the capsular polysaccharide (K-antigen) locus of *Porphyromonas gingivalis*. *Infection and Immunity* 2006;**74**(1):449–60.
21. Mollenhauer HH, Morré DJ, Rowe LD. Alteration of intracellular traffic by monensin; mechanism, specificity and relationship to toxicity. *Biochimica et Biophysica Acta* 1990;**1031**(2):225–46.
22. van Eijk LT, Dorresteijn MJ, Smits P, van der Hoeven JG, Netea MG, Pickkers P. Gender differences in the innate immune response and vascular reactivity following the administration of endotoxin to human volunteers. *Critical Care Medicine* 2007;**35**(6):1464–9.
23. Bouman A, Moes H, Heineman MJ, de Leij LF, Faas MM. The immune response during the luteal phase of the ovarian cycle: increasing sensitivity of human monocytes to endotoxin. *Fertility and Sterility* 2001;**76**(3):555–9.
24. van der Velden U. The Dutch periodontal screening index validation and its application in The Netherlands. *Journal of Clinical Periodontology* 2009;**36**(12):1018–24.
25. Sánchez-Hérnandez PE, Zamora-Perez AL, Fuentes-Lerma M, Robles-Gómez C, Mariaud-Schmidt RP, Guerrero-Velázquez C. IL-12 and IL-18 levels in serum and gingival tissue in aggressive and chronic periodontitis. *Oral Diseases* 2011;**17**(5):522–9.
26. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. *Nature Reviews Immunology* 2003;**3**(2):133–46.
27. Gabay C. Interleukin-6 and chronic inflammation. *Arthritis Research and Therapy* 2006;**8**(Suppl. 2):S3.
28. Okada H, Murakami S. Cytokine expression in periodontal health and disease. *Critical Reviews in Oral Biology and Medicine* 1998;**9**(3):248–66.
29. Nakano K, Kuboniwa M, Nakagawa I, Yamamura T, Nomura R, Okahashi N, et al. Comparison of inflammatory changes caused by *Porphyromonas gingivalis* with distinct *fimA* genotypes in a mouse abscess model. *Oral Microbiology and Immunology* 2004;**19**(3):205–9.
30. Potempa J, Banbula A, Travis J. Role of bacterial proteinases in matrix destruction and modulation of host responses. *Periodontology 2000* 2000;**24**:153–92.
31. Singh A, Wyant T, Anaya-Bergman C, Aduse-Opoku J, Brunner J, Laine ML, et al. The capsule of *Porphyromonas gingivalis* leads to a reduction in the host inflammatory response, evasion of phagocytosis, and increase in virulence. *Infection and Immunity* 2011;**79**(11): 4533–42.
32. Brunner J, Scheres N, El Idrissi NB, Deng DM, Laine ML, van Winkelhoff AJ, et al. The capsule of *Porphyromonas gingivalis* reduces the immune response of human gingival fibroblasts. *BMC Microbiology* 2010;**10**:5.
33. Bramanti TE, Wong GG, Weintraub ST, Holt SC. Chemical characterization and biologic properties of lipopolysaccharide from *Bacteroides gingivalis* strains W50, W83, and ATCC 33277. *Oral Microbiology and Immunology* 1989;**4**(4):183–92.
34. Ogawa T, Asai Y, Makimura Y, Tamai R. Chemical structure and immunobiological activity of *Porphyromonas gingivalis* lipid A. *Frontiers in Bioscience* 2007;**12**:3795–812.

35. Kikkert R, Laine ML, Aarden LA, van Winkelhoff AJ. Activation of Toll-like receptors 2 and 4 by gram-negative periodontal bacteria. *Oral Microbiology and Immunology* 2007;**22**(3):145–51.
36. Dixon DR, Darveau RP. Lipopolysaccharide heterogeneity: innate host responses to bacterial modification of lipid a structure. *Journal of Dental Research* 2005;**84**(7):584–95.
37. Bainbridge BW, Darveau RP. *Porphyromonas gingivalis* lipopolysaccharide: an unusual pattern recognition receptor ligand for the innate host defense system. *Acta Odontologica Scandinavica* 2001;**59**(3):131–8.
38. Manicassamy S, Pulendran B. Modulation of adaptive immunity with Toll-like receptors. *Seminars in Immunology* 2009;**21**(4):185–93.
39. Sieling PA, Chung W, Duong BT, Godowski PJ, Modlin RL. Toll-like receptor 2 ligands as adjuvants for human Th1 responses. *Journal of Immunology* 2003;**170**(1): 194–200.
40. Mele T, Madrenas J. TLR2 signalling: at the crossroads of commensalism, invasive infections and toxic shock syndrome by *Staphylococcus aureus*. *International Journal of Biochemistry and Cell Biology* 2010;**42**(7):1066–71.